STRUCTURAL CHARACTERIZATION OF SEVEN DIFFERENT SUBUNITS IN *ANDROCTONUS AUSTRALIS* HAEMOCYANIN

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1. Introduction

Arthropod haemocyanins are very large oligomeric copper containing proteins. They are made up of many components, usually designated as monomers, each of which has a relative molecular mass of about 75 000. In the scorpion *Androctonus australis,* the native molecule has a sedimentation coefficient of 34 S corresponding to 24 subunits and appears in electron microscopy to be constituted of two rectangular structures linked together by bridges. It has been shown [1] that after extensive dialysis, 1 M urea produces almost complete dissociation of the 34 S molecule into 6 electrophoretically resolvable fractions. From the latter, 8 antigenically distinct monomers could be isolated, easily identifiable by immunochemistry [2].

Several reasons such as antigenicity [2], structural role in reassembly experiments by calcium [1], chromatographic and electrophoretic behaviour, indirectly were in favour of the existence of 8 distinct monomers. The latter also exhibited a lot of common properties such as molecular weight, globular structure in electron microscopy, ultraviolet absorption spectrum, copper content, 1 oxygen binding site/molecule: thus the possibility that at least some of them represented different conformations of the same molecule could not be ruled out.

N-terminal sequence determination was undertaken in order to ascertain the purity of the different isolated polypeptide chains and to investigate their possible structural relationship. It was also probed whether subunits of a phylogenically related species, namely the horseshoe crab *Limulus polyphemus,* were structurally similar or not.

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2. Materials and methods

Subunits of *Androctonus australis* haemocyanin were isolated and purified according to [2]. Fraction IV of *Limulus polyphemus* haemocyanin was a gift from Dr J. Bonaventura. The antigenic purity of each monomer was checked by the crossed immunoelectrophoresis technique [3] using an antiserum specific for the unfractionated mixture of the haemocyanin dissociation products. All the subunits yielded unique and almost perfectly symmetrical peaks.

All reagents (analytical grade) were obtained from Merck or Prolabo except those employed for the Sequencer which were purchased from SDS (Marseilles).

Automated Edman degradation was carried out in a Beckman Sequencer Model 890 C by the 0.33 M quadrol double cleavage method. The thiazolinones were converted into phenylthiohydantoin (PTH)-amino acids by treatment with 20% trifluoroacetic acid at 80°C for 12 min [4]. The PTH-amino acids were characterized by thin-layer chromatography (chloroform–methanol, 90:10, (v/v); pure chloroform) and by high-performance liquid chromatography (Waters chromatograph, Model ALC/GPC-204) on a 30 cm Waters μ-Bondapak C 18 column as in [5].

3. Results

The 8 monomers of *Androctonus australis* haemo-

Table 1
Automatic Edman degradation of subunits 3C (38 nM) and 5A (38 nM) of the haemocyanin of the scorpion *Androctonus australis*

Cycle	3C		5A	
	a	b	a	b
1	Ala	Ala (66)	Gly	Gly (59)
2	Pro	Pro (43)	Val or Phe or Met	Val (43)
3	Leus[c]	Ile (85)	Gln and Glu	Gln (22) and Glu (21)
4	Asn	Asn (70) and Asp (24)	Asp or Arg	Asp (53)
5	Leus	Ile (81)	Lys or Trp	Lys (56)
6	Gln and Glu	Gln (31) and Glu (18)	Gln and Glu	Gln (12) and Glu (10)
7	Asp or Arg	Arg (58)	Glu	Glu (12)
8	Asp or Arg	Arg (62)	Asp or Arg	Arg (21)
9	Leus	Ile (24)	Leus	Leu (20)
10	Leus	Leu (32)	Leus	Leu (23)
11	Ser	Ser (20)	Pro	Pro (7)
12	Leus	Leu (26)	Leus	Leu (17)
13	Val or Phe or Met	Phe (12)	Val or Phe or Met	Phe (7)
14	Glu	Glu (6)	Asp or Arg	Asp (3)

[a] Thin layer chromatography
[b] High-performance liquid chromatography, results give the percentage
[c] Leus, Leu or Ile

cyanin, 2, 3A, 3B, 3C, 4, 5A, 5B and 6 according to [2] and fraction IV of *Limulus polyphemus* haemocyanin were submitted to automated Edman degradation at 35–40 nM. As an example, all the details concerning the establishment of the N-terminal sequences of subunits 3C and 5A are indicated in table 1. Table 2 gives a comprehensive view of the results so far obtained.

Seven subunits (2, 3B, 3C, 4, 5A, 5B, 6) were pure polypeptide chains as only one amino acid could be characterized at each degradation step. They all possessed different primary structures. They have

Table 2
N-terminal sequences of seven subunits (2, 3B, 3C, 4, 5A, 5B, 6) of the haemocyanin of the scorpion *Androctonus australis* and of fraction IV of the horseshoe crab *Limulus polyphemus* haemocyanin

Subunit \ Cycle	−2	−1	1	2	3	4	5	6	7	8	9	10	11	12	13	14
2			Thr	Val	Lys	Glu	Lys	Gln	Asp	Arg	Ile	Ile	Pro	Leu	Phe	Glu
4			Thr	Val	Lys	Glu	Lys	Gln	Gln	X	Leus[a]	Leus	Ser	Leus	Phe	Lys
6			Thr	Val	Ala	Asp	Lys	X[b]	Ala	Arg	Leu	X	Pro	Leu		
5A			Gly	Val	Gln	Asp	Lys	Gln	Glu	Arg	Leu	Leu	Pro	Leu	Phe	Asp
3B			X	Leu	His	Glu	Lys	Gln	Ile	Arg	Ile	Leu	Lys	Leu	Phe	Lys
5B	Thr	Leu	Asn	Ile	Glu	Glu	Lys	X	X	X	Leu	Leu				
3C			Ala	Pro	Ile	Asn	Ile	Gln	Arg	Arg	Ile	Leu	Ser	Leu	Phe	Glu
IV			Thr	Leu	Lys	Glu	Lys	Gln	Asp	X	Ile	Leu				

[a] Leus, Leu or Ile
[b] X, amino acid not yet characterized

many common points in their N-terminal parts: Thr was the N-terminal amino acid of 4 of them (2, 4, 5B, 6). Six subunits had Leu, Val, or Ile in position 2, Glu or Asp in position 4 and Lys in position 5. Gln was in position 6, Arg in position 8, Leu or Ile in position 10, Leu in position 12 and Phe in position 13 in all the subunits where an amino acid was characterized in these positions. Finally Leu or Ile was found in position 9 in all the subunits. The close homology of subunits 2 and 4 should more particularly be pointed out.

The N-terminal amino acid of fraction 3B could not be so far identified neither by the above quoted technique nor by the dansylation procedure; however leucine was always found only at the second degradation step. Fraction 3A seemed quite labile and could not be sequenced: indeed several amino acids were characterized at each step. It was probably degraded during the extensive dialysis and/or the lyophilisation occurring prior to the sequence determination.

4. Discussion

This report definitively establishes by a direct method that *Androctonus australis* haemocyanin contains 7 pure polypeptide chains. The existence of an eighth was discussed above and seems probable. The heterogeneity of the haemocyanin constituents had been demonstrated by several methods such as ion exchange chromatography [6], sodium dodecylsulfate polyacrylamide gel electrophoresis [7], immunochemistry [2], genetics [8] and peptide mapping [9] but this work is, to our knowledge, the first to prove that the sequences of the polypeptide chains are different at least in their N-terminal parts.

A rather large homology appeared between the N-terminal sequences of 7 subunits suggesting that they might arise from a common ancestor. This data support their common ability to reversibly bind oxygen and their similar copper content and ultraviolet absorption spectrum. Subunits 3C and 5B resembled all the others except for the 5 first amino acids.

Subunit 3A had a different behaviour from the 7 other fractions because of its great lability.

A strong homology occurred also between subunit IV from *Limulus polyphemus* haemocyanin and subunits 2 and 4 from *Androctonus australis* haemocyanin. These two phylogenically related arthropod species belong to the class of Chelicerata. It is remarkable that, in their N-terminal parts, subunits 2 and 4 from *Androctonus australis* are more similar to the *Limulus* IV fraction than to the other *Androctonus* subunits. This might be in favour of a relatedness between the N-terminal parts of these subunits and a structural or functional advantage which could be responsible for the preservation of this sequence during evolution.

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